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(54) Title: KERATINOCYTE-DERIVED CONDITIONED MEDIUM AS A SOURCE OF GROWTH FACTORS

(57) Abstract

Human epithelial cells are cultured in protein-free standard media to produce autocrine- and paracrine-acting growth factors. The resulting mixture of human keratinocyte-derived conditioned medium factors (kdCMF) is useful to promote healing of surface wounds, ulcerations and other hypoproliferative skin pathologies.

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KERATINOCYTE-DERIVED CONDITIONED MEDIUM AS A SOURCE OF GROWTH FACTORS

Technical Field

This invention is in the general field of wound healing. More specifically it relates to a novel method of producing human keratinocyte-derived conditioned medium factors (kdCMF) which promote healing of surface wounds, ulcerations, and other hypoproliferative skin conditions.

Background of the Invention

The growth of cells may be regulated by both autocrine and paracrine growth factors. Autocrine growth factors are those secreted by growing cells which factors stimulate or inhibit the proliferation of the same cell. Paracrine growth factors are those that act on neighboring cells.

It was originally believed that the growth of normal cells was controlled by paracrine factors while the growth of malignant cells was promoted by autocrine factors. More recently, exceptions to these generalizations have become evident as autocrine growth factors have been implicated in the normal regulation of cell proliferation during growth, development and wound repair (Clark and Henson (eds), The Molecular and Cellular Biology of Wound Repair (1988); Barbul et al. (eds) Progress in Clinical and Biological Research (1987), Vol. 266. Indeed, it has been shown by the inventors herein that normal keratinocytes can be cultured in a growth factor-free medium in vitro

(Shipley, G. et al., J Cell Physiol (1989) 138:511-518; Cook, P. et al., J Cell Physiol (1991) 146:277-289).

Studies have suggested that neonatal human keratinocytes produce autocrine- or paracrine-acting growth factors in vitro (Gilchrest et al., J Cell Physiol (1983) 117:235-240; Cook et al., J Cell Physiol (1991) 146:277-289). More specifically, it has been shown that human keratinocyte cultures produce TGFα (Coffey et al., Nature (1987) 328:817-820; Pittelkow et al., J Biol Chem (1989) 264:5164-5171; Cook et al., Mol Endocrin (1990) 4:1377-1385), keratinocyte-derived autocrine factor (KAF)/amphiregulin (AR) (Cook et al., Mol Cell Biol (1991) 11:2547-2557, TGFβ (Shipley et al., Cancer Res (1986) 46:2068-2071) and bFGF (Cook et al., Mol Endocrin (1990) 4:1377-1385). Other investigators have also demonstrated that human keratinocytes produce bFGF (Halaban et al., J Cell Biol (1988) 107:1611-1619), PDGF (Ansel et al., J Invest Dermatol (1990) 94:101s-107s) and interleukins (Blanton et al., Proc Natl Acad Sci USA (1989) 86:1273-1277; Kirnbauer et al., J Immunol (1989) 142:1922-1928).

It is known that damage to the human skin results in the expression of growth factors at the wound site (Clark and Henson (eds), The Molecular and Cellular Biology of Wound Repair (1988); Barbul et al. (eds), Progress in Clinical and Biological Research (1987) Vol. 266; Rappolee et al., Science (1988) 241:708-712; Antoniades et al., Proc Natl Acad Sci USA (1991) 88:565-569); and various approaches to the use of factors provided by cells in the healing of wounds have been tried.

First, several individual growth factors have been shown to modulate the wound healing process (Clark and Henson (eds), The Molecular and Cellular Biology of Wound Repair (1988); Pierce et al., J Cell Biol (1989)

109:429-440; Barbul et al. (eds), Progress in Clinical and Biological Research (1987), Vol. 266.

Second, it has also been attempted to use whole epithelial cells in wound healing. Application of allogenic keratinocyte grafts to surface wounds and ulcerations in humans functions to promote the healing of these lesions has shown positive results (Phillips et al., Clin Res (1988) 36:684A; Burt et al., Br Med J (1989) 298: 915-917; Brian et al., Br Med J (1989) 298:917-919; Phillips and Gilchrest, Dermatol Surg Oncol (1989) 15:1169-1176; Phillips et al. J Am Acad Dermatol (1989) 21:191-199). However, the treatment of wounds with allogenic cells or individual growth factors includes inherent problems concerning safety and efficacy. The present invention endeavors to overcome such problems.

Disclosure of the Invention

The present invention involves the preparation and use of a mixture of growth factors, both autocrine and paracrine, produced by human epithelial cells. This represents a desirable combination of factors capable of effecting wound healing and tissue repair. Such combinations of factors in their native proportion are particularly desirable for therapeutic purposes and can be so used by itself or in a pharmaceutical formulation. This mixture of growth factors is produced by harvesting conditioned medium from cultures of human epithelial cells grown in protein-free medium.

In one aspect, the invention relates to a method to produce human keratinocyte-derived conditioned medium factors (kdCMF), which method comprises culturing human epithelial cells in a protein-free medium to obtain a conditioned medium and recovering the conditioned medium from the culture.

Another aspect of the invention relates to the use of kdCMF to promote healing of surface wounds, ulcerations and other hypoproliferative skin pathologies in humans, and to pharmaceutical compositions useful for this purpose.

Still another aspect of the invention relates to the use of kdCMF as a supplement to cell culture medium for the purpose of promoting cell growth and viability of cells other than human keratinocytes.

An object of the invention is to provide a novel mixture of growth factors which growth factors include autocrine and paracrine growth factors which growth factors are present with respect to each other in substantially the same relative amounts in which they naturally occur indigenously within a mammal such as a human.

An advantage of the present invention is that the mixture of growth factors provides efficacious results when applied to a wound in terms of increasing the rate of wound healing.

A feature of the present invention is that the mixture of growth factors is non-toxic in that it includes naturally occurring growth factors in their naturally occurring proportional amounts.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the means of production and use as fully set forth below with reference being made to the accompanying examples forming a part hereof.

Detailed Description of the Invention

Before the present mixture of growth factors and methods of preparing and using such are described, it is to be understood that this invention is not limited to

the particular species (humans), cell types (human keratinocytes) or culture mediums described as such may, of course, vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a mixture" of growth factors may include a plurality of different mixtures of growth factors obtained from different sources, reference to "an autocrine" refers to one or more autocrine growth factor compounds alone or in combination with each other and reference to "the protein-standard medium" includes any such medium of the type described herein and of the type which would become apparent to those skilled in the art upon reading this disclosure, and so forth.

The invention is directed to the use of "keratinocyte-conditioned medium factors" (kdCMF). As defined herein, kdCMF refers to the mixture of autocrine and paracrine factors which are produced by culturing human epithelial cells in a protein-free medium. Thus, when such epithelial cells are allowed to grow in a protein-free medium, the medium will contain a mixture of these factors produced by the cells. In its simplest form, the kdCMF of the invention is simply the conditioned medium harvested from such cultures.

The conditioned medium itself can also be further fractionated (using standard separation protocols based on charge, hydrophobicity, size or ligand affinity) into two or several fractions which retain mixtures of the secreted factors, although in altered ratio. Such

crude fractions can also be used in the wound-healing methods and cell proliferation methods of the invention and are another embodiment of kdCMF.

The conditioned medium can also be concentrated and/or freed of inorganic and small organic materials by dialysis and/or lyophilization and/or other methodologies. The resulting concentrated form of the conditioned medium also contains these factors, and is thus a more convenient form of kdCMF.

In its preferred form, the invention involves the purified mixture of growth factors which are obtained from the protein-free medium on which the epithelial cells are allowed to grow. The growth factors are purified but maintained so that the natural ratio of each of the growth factors to each other remains substantially undisturbed. It is believed that by maintaining the particular ratio of each growth factor to the other, it is possible to obtain a mixture of growth factors which is both safe and efficacious with respect to enhancing the rate of wound healing.

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

As used herein, the term "defined medium" refers to medium which contains no crude extracts or common supplements such as pituitary extract, serum proteins, and so forth. "Defined medium" refers to the conventional understanding as it pertains to growth of cells in a medium containing no undefined additives, as that term is commonly used in the art. In the present invention, however, "protein-free standard medium" also lacks exogenous artificial sources of specific growth factors such as EGF, TGFα, or other polypeptides. One illustrative embodiment of "protein-free standard medium" is described herein.

The term "complete medium" refers to high amino-acid (HAA) modified medium MCDB 153 (Pittelkow and Scott, Mayo Clin Proc (1986) 61:771-777, incorporated herein by reference to disclose such a medium) supplemented with 0.2% (v/v) bovine pituitary extract (BPE), culture grade EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5 x 10^{-7} M), ethanolamine (1 x 10^{-4} M) and phosphoethanolamine (1 x 10^{-4} M), gentamicin sulfate (μg/ml) or in KBM medium (Clonetics Corp.) with the same supplements.

One form of "defined medium" illustrated herein as "defined medium-1" refers to complete medium without BPE.

"Protein-free standard medium" as illustrated herein as "protein-free standard medium-1" refers to complete medium without BPE, EGF and insulin.

The kdCMF can be produced using a variety of protocols which are similar to that described in Example 1 herein, and generally by plating human epithelial cells in complete, defined or protein-free standard medium at varying densities in different cell culture vessels or on different substrates. Alternative protocols utilize other complete, defined or protein-free media to plate the human keratinocytes. Once plated, the medium is changed to protein-free standard medium to facilitate the isolation of kdCMF. While human keratinocytes are preferred, related cell types (other human epithelial cells) which may or may not be immortalized are viable alternatives in the generation of kdCMF.

Although the preferred embodiment of the present invention involves the use of human epithelial cells and more specifically human keratinocytes, the present invention is intended to encompass other mixtures of growth factors which can be obtained from the culturing of non-human mammalian epithelial cells and

non-human mammalian keratinocytes. As will be apparent to those skilled in the art, certain modifications to the medium upon which those cells are grown should be made in order to allow for the efficient growth of such non-human cells. It may be most desirable to obtain certain non-human cells such as porcine epithelial cells or porcine keratinocytes in that the growth factors and growth factor mixtures produced by such cells might well be readily amenable for human use. Accordingly, the present invention includes mixtures of human and non-human growth factors obtained from the culturing of human and non-human epithelial cells and specifically human and non-human keratinocytes, as well as pharmaceutical formulations containing such mixtures of growth factors and methods of using such mixtures and formulations in order to promote wound healing.

Wound Healing

The human skin consists of a vascularized dermis that is separated by a basement membrane from the avascular epidermis. The epidermis is composed of several topologically organized compartments including a proliferative basal layer and post-mitotic suprabasal layers which differentiate and form the keratinized outer layer of the skin. It is generally regarded that proliferating keratinocytes within the basal layer of the epidermis must rely on the vascularized dermal layers for nutritive support.

The wound-healing process is clearly a complicated sequence of events which may involve neovascularization, synthesis of extracellular matrix components and stimulation of cell migration and proliferation. The exact identities and quantities of factors necessary for expedient healing of surface wounds in humans are not known, and all of the factors which

regulate the processes of wound-healing are not completely understood. Thus, the application of single growth factors (FGFs, EGF, TGFα, PDGFs, TGFβs) to promote the healing of wounds and ulcerations is of less benefit than the addition of preparations which contain factors that more closely mimic the array and concentration of factors produced by epidermal cells at the site of injury. Autonomously proliferating normal human keratinocyte cultures are analogous to wounded epidermis and therefore conditioned media from these cells will contain an array of wound healing/stimulating factors that will be of value in treating a variety of surface wounds, ulcerations and hypoproliferative pathologies in humans.

The application of kdCMF to surface wounds and epidermal pathologies in humans is an improvement over existing technologies. Unlike preparations containing a single purified growth factor, kdCMF is a complete mixture of keratinocyte-derived factors capable of expediting the wound-healing process in a manner similar to that mediated by epidermal cells at the site of injury. kdCMF also offers advantages over the use of keratinocytes per se. Exposure to viral pathogens which may be present in the live cells derived from allogenic donors is avoided as well, as is the exposure of patients to undefined antigenic contaminants (bovine serum, murine cell lines) associated with other methods currently used to culture human keratinocytes (Rheinwald and Green, Cell (1975) 6:331-344). This avoids the possibility of inducing serum sickness or other undesirable immunological reactions (Meyer et al., H Trauma (1988) 28:1054-1059; Johnson et al., J Burn Care Rehabil (1990) 11:504-509).

The conditions which may benefit from the application of kdCMF include, but are not limited to:

epidermal ulcerations (decubitus ulcers, ischemic ulcers, infarctive ulcers, vascular ulcers, and hemoreologic ulcers), surface wounds (thermal and chemical induced burns, abrasions, lacerations, incisions, skin graft donor sites, and skin graft recipient sites), lupus erythematosus, corticosteroid-induced atrophy, pemphigus, pemphigoid, androgenetic alopecia and alopecia areata.

For application of the kdCMF of the invention therapeutically, the active ingredient is formulated into suitable topical compositions, including salves, creams, lotions, solutions, and the like. Additional excipients and palliative factors may also be added. Formulations suitable for topical compositions are well known in the art and may be found, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA.

The compositions useful in wound healing may also include autologous cells (normal human keratinocytes and/or fibroblasts) or other purified growth factors (TGFα, TGFβ, FGFs, HB-EGF, EGF, AR, PDGFs, epithelins). Simultaneous or additional administration of these cells and/or factors will be of benefit in mediating the wound-healing process. Moreover, application of kdCMF with or without the above-described additives with bandages, or with solid matrices or supports (collagen, collagen-glycosaminoglycan) will be of value as effective methods of delivering kdCMF to lesions and wounds.

In addition to therapeutic use, the kdCMF of the invention may be used as a supplement in culturing various cells which can benefit from this array of growth factors. Suitable cell types which can be benefited in this way include various epithelial cultures, such as those derived from the skin trachea, bronchia, urogenital tract and mammary epithelium.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the growth factor mixtures of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, time, etc.), but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade and pressure is at or near atmospheric.

Preparation A

Preparation of Human Keratinocyte Cultures

Primary cultures of human keratinocytes can be isolated by the trypsin float technique as described in Wille et al., J Cell Physiol (1984) 121:31-34, or other suitable methods. Stock cultures can be maintained (Shipley et al., J Cell Physiol (1989) 138:511-518; Shipley and Pittelkow, Arch Dermatol (1987) 123:1541a-1544a) in an actively growing state in a complete or defined medium.

Example 1

Preparation of kdCMF

Keratinocytes are plated in complete or defined medium at a density of 1-5 x 10^3 cells/cm^2 in tissue culture dishes or other suitable vessels. After 1-3 days of incubation, the cells are washed 3 times with Hepes buffered normal saline and the medium replaced with protein-free standard medium. After 24 hours of additional incubation, the medium is discarded and replaced with fresh protein-free standard medium.

Protein-free standard medium conditioned by human keratinocytes is collected every 24-48 hours for 4-5 days until the cells reach 90-95% confluency. Keratinocyte conditioned medium (CM) is collected and frozen at -80°C. CM can be later thawed, pooled and concentrated. In addition to the original components of protein-free standard medium, concentrated keratinocyte-derived CM contains kdCMF.

The bioactivity of kdCMF is assayed on any cell type which responds to the factors present in kdCMF. AKR-2B, NR6, human dermal fibroblasts, Balb/MK, and human keratinocytes at clonal density are used to detect kdCMF bioactivity (Cook et al., J Cell Physiol (1991) 146:277-289).

The instant invention is shown and described herein in what is considered to be the most practical and preferred embodiments with respect to the growth factor mixtures, their methods of preparation, formulation and use. It is recognized, however, that departures may be made therefrom which are within the scope of the invention and that modifications will occur to one skilled in the art upon reading this disclosure.

Claims

1. A method of preparing mammalian keratinocyte-derived conditioned medium factors (kdCMF) comprising:

a) culturing mammalian epithelial cells in a protein-free standard medium to obtain a conditioned medium containing said factors; and

b) recovering the conditioned medium from the culture.

2. The method of claim 1, wherein the epithelial cells are keratinocytes.

3. The method as claimed in claim 1, wherein the epithelial cells are human epithelial cells.

4. The method as claimed in claim 3, wherein the epithelial cells are human keratinocytes.

5. The method of claim 1, further comprising:

fractionating said conditioned medium and recovering a fraction thereof, wherein said fractionating is conducted by separations based on charge, hydrophobicity, size or ligand affinity.

6. The method of claim 1, further comprising concentrating the kdCMF from said conditioned medium using dialysis.

7. The method of claim 5, further comprising lyophilizing the kdCMF.

8. A method to promote the healing of surface wounds in humans which comprises applying the kdCMF of claim 1 to the surface wound.

9. The method of claim 8, wherein the surface wound is a thermal or chemical induced burn, an abrasion, laceration or incision, or a skin graft donor or recipient site.

10. The method of claim 8, wherein the kdCMF is applied in the form of a solution, salve, lotion, or creme.

11. A method to promote the healing of surface ulcerations or hypoproliferative skin pathologies in humans which comprises applying the kdCMF of claim 1 to the surface ulcerations or hypoproliferative skin pathologies.

12. The method of claim 11, wherein the surface ulceration is a decubitus, ischemic, infarctive, vascular or hemoreologic ulcer.

13. The method of claim 11, wherein the hypoproliferative skin pathology is lupus erythematosus, corticosteroid-induced atrophy, pemphigus, pemphigoid, androgenic alopecia or alopecia areata.

14. The method of claim 11, wherein the kdCMF is applied in the form of a solution, salve, lotion, or creme.

15. A method to promote cell growth and viability of responsive mammalian cells in culture which comprises providing to said culture the kdCMF of claim 1.

16. A pharmaceutical composition which comprises the kdCMF of claim 1 in admixture with at least one pharmaceutical excipient.

17. The composition of claim 16, which further comprises growth factors.

18. The composition of claim 16, which further comprises normal human keratinocytes or fibroblasts.

19. A medical article which comprises a solid substrate that includes the kdCMF of claim 1.

20. The article of claim 19, wherein said substrate is a bandage.

21. The article of claim 17, wherein said substrate is a collagen or collagen-GAG-containing matrix.

22. The article of claim 17, wherein said substrate is an artificial biocompatible matrix.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06161

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00
U.S.CL.: 435/240.2

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/240.1; 240.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Biosis "Pratein Free Medin", AU= "Shipley G or Shipley G D", "Growth factor", "Conditioned"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	01 May Cancer Research, Volume 49, issued 1989, D. Boyd et al., "Examination of the Effects of Epidermal Growth Factor on the Production of Urokinase and the Expression of the Plasminogen Activator Receptor in a Human Colon Cancer Cell Line," pages 2427-2432, see entire document.	1-7
Y	Journal of Cellular Physiology, Volume 140, issued 1989, Pittelkow et al., "Serum-Free Culture of Normal Human Melanocytes: Growth Kinetics and Growth Factor Requirements," pages 565-576, see entire document.	1-7
Y Y	Journal of Cellular Physiology, Volume 138, issued 1989, G.D. Shipley, et al., "Growth of Normal Human Keratinocytes and Fibroblasts in Serum-Free Medium Is Stimulated by Acidic and Basic Fibroblast Growth Factor," pages 511-518, see entire document.	8-14 15-22

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 DECEMBER 1991	26 DEC 1991
International Searching Authority	**Signature of Authorized Officer**
RO/US	Jane Williams for Diane Moffett

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications, published 1988 by Alan R. Liss, Inc. (New York), G.G. Nemeth et al., pages 1-17, see entire document.	8-14 16-22
Y Y	Journal of Surgical Research, Volume 43, issued 1987, A. Buckley et al., "Epidermal Growth Factor Increases Granulation Tissue Formation Dose Dependently," pages 322-328, see entire document.	8-14 16-22
Y Y	International Journal of Tissue Reactivity, Volume X, Number 6. issued 1988, G.R. Grotendorst, "Growth Factors as Regulators of Wound Repair," pages 337-344, see entire document.	8-14 16-22
Y Y	Proceedings of the National Academy of Sciences, Volume 85, issued March 1988, M. Eisinger et al., "Growth Regulation of Skin Cells by Epidermal Cell-Derived Factors: Implications for Wound Healing," pages 1937-1941, see entire document.	8-14 16-22
Y	Tissue Culture Association 41st Annual Meeting, Houston TX, June 10-13, 1990, Annal Meeting Abstracts, issued 1990, G.D. Shipley., "Culture of Human Cells in Optimized Media," page 24A, see entire document.	8-18
Y	US, A, 4,423,145 (STAMPFER et al.) 27 December 1983, see entire document.	15